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## Short communication

## Production and characterisation of monoclonal antibodies specific for chicken interleukin-12

S. Balu<sup>1</sup>, L. Rothwell\*, P. Kaiser<sup>2</sup>

Institute for Animal Health, Compton, Berkshire RG20 7NN, UK

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## ABSTRACT

Using genetic immunisation of mice, we produced antibodies against chicken interleukin-12p40 (chIL-12p40), also known as IL-12 $\beta$ . After a final injection with a recombinant chIL-12p40 protein, several stable hybridoma cell lines were established which secreted monoclonal antibodies (mAbs) to this component of the heterodimeric IL-12 cytokine. Specific binding of three of the mAbs to COS-7 cell-derived recombinant chIL-12p40 and the chIL-12p70 heterodimer was demonstrated in an indirect ELISA, and in dot blots. Two of the mAbs were used to develop a capture ELISA, suitable for detecting both recombinant protein (chIL-12p40 and the heterodimeric p70 protein) and native chIL-12. The mAbs were further characterised to show utility in immunocytochemistry.

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## 1. Introduction

Interleukin-12 (IL-12) is a 70 kDa heterodimeric cytokine, comprising IL-12p35 (IL-12 $\alpha$ ) and IL-12p40 (IL-12 $\beta$ ), which plays a crucial role in the initiation and progression of a Th1-type response (reviewed by Trinchieri, 2003). In infections caused by bacteria and intracellular parasites, IL-12 is produced by antigen-presenting cells within a few hours of infection. It acts as a pro-inflammatory cytokine, activating NK cells (Chan et al., 1991) and inducing IFN- $\gamma$  production, which in turn enhances phagocytic and bactericidal activity of phagocytic cells and their ability to release IL-12 and other pro-inflammatory cytokines. Furthermore, IL-12 produced during the early stages of infection or inflammation deter-

mines the ensuing antigen-specific immune response, favouring differentiation of CD4<sup>+</sup> T cells into Th1 cells, whilst inhibiting differentiation of Th2 cells through the resulting production of IFN- $\gamma$ .

Until relatively recently, progress in understanding and dissecting the avian immune response to a variety of pathogens has been hampered by the lack of cross-reactivity and low level of sequence homology between avian and mammalian immune-related molecules. However, the availability of the chicken genome (International Chicken Genome Sequencing Consortium, 2004) has provided a unique resource to further our understanding of the general biology and more specifically the immune-related genes in the chicken (Kaiser et al., 2005). We are now able to measure expression of many genes, including most cytokines, at the mRNA level by real-time quantitative RT-PCR (qRT-PCR) (Eldaghyes et al., 2006; Kaiser and Stäheli, 2008); however, to date, there still remains a paucity of reagents available to measure the immune response in the chicken, particularly at the protein level. Indeed, although the two sub-units of the functional chicken IL-12p70 molecule were cloned some years ago (Balu and Kaiser, 2003; Degen et al., 2004), the only assays available to measure chicken IL-12 (chIL-12) at the protein level are

\* Corresponding author at: The Roslin Institute & R(D)SVS, University of Edinburgh, Easter Bush Veterinary Centre, Midlothian EH25 9RG, UK. Tel.: +44 131 650 6245; fax: +44 131 651 3903.

E-mail address: [lisa.rothwell@roslin.ed.ac.uk](mailto:lisa.rothwell@roslin.ed.ac.uk) (L. Rothwell).

<sup>1</sup> Current address: Clinical and Diagnostic Sciences Group, Guy's Campus of Kings College, London, UK.

<sup>2</sup> Current address: The Roslin Institute & R(D)SVS, University of Edinburgh, Easter Bush Veterinary Centre, Midlothian EH25 9RG, UK.

an indirect bioassay based on the stimulation of IFN- $\gamma$  synthesis or a non-specific assay measuring the proliferation of splenocytes (Degen et al., 2004).

The aim of this study was to generate monoclonal antibodies (mAbs) to chIL-12 for use in assays to specifically measure IL-12 production. Using a pair of the anti-chIL-12 mAbs generated, a capture ELISA was developed that could detect natural and recombinant chIL-12. The mAbs also show utility in immunocytochemistry.

## 2. Materials and methods

### 2.1. IL-12 expression

A *NotI* fragment containing the chIL-12p40 cDNA (Balu and Kaiser, 2003) was inserted into the *NotI* site in the polylinker of the eukaryotic expression vector pCI-neo (Promega, Southampton, UK) giving rise to pCI-neo-chIL-12p40.

ChFlexi-IL-12, a single-chain chIL-12p35-p40 heterodimeric construct cloned into pcDNA3.1 (Invitrogen, Paisley, Scotland) was a kind gift from W.G.J. Degen, Intervet International BV, Boxmeer, The Netherlands (Degen et al., 2004).

Both constructs were expressed in COS-7 cells (ex-COS) using a well-described DEAE-dextran transfection method (Lawson et al., 2000, 2001; Rothwell et al., 2004; Tregaskes and Young, 1997).

### 2.2. Monoclonal antibody production, purification and labelling

MABs were generated following intramuscular genetic immunisation of mice with pCI-neo-chIL-12p40 (100  $\mu$ g endotoxin-free DNA/mouse in a total volume of 100  $\mu$ l PBS, 50  $\mu$ g/hind quadriceps), following a previously described procedure (Rothwell et al., 2001). Following the fusion, hybridoma supernatants were screened for antibodies to chIL-12p40 by indirect ELISA, described below. Positive hybridomas were selected for cloning (by limiting dilution) and expansion. The isotype of each mAb was determined using the IsoStrip mouse mAb isotyping kit (Roche Diagnostics, Burgess Hill, UK), according to the manufacturer's instructions.

MABs were purified using HiTrap Protein G columns (GE Healthcare Life Sciences, Little Chalfont, UK) according to the manufacturer's instructions. Concentrations of purified mAbs were determined by absorbance at 280 nm, and then dialysed extensively against PBS using 10,000 Da molecular weight cut-off (MWCO) Slide-A-Lyser cassettes (Perbio Science, Tattenhall, UK).

Purified mAbs were conjugated to biotin by incubating with ImmunoPure Sulfo-NHS-LC-Biotin (Perbio Science) for 2 h at room temperature (RT), as per the manufacturer's instructions. Biotinylated mAbs were again dialysed against PBS using 10,000 Da MWCO Slide-A-Lyser cassettes.

### 2.3. Antibody assays

Indirect ELISA was performed as described previously (Rothwell et al., 2001). Briefly, supernatant from fusion

wells (100  $\mu$ l) or 50  $\mu$ l of mouse serum diluted 1:200 in PBS containing 0.05% Tween 20 (PBS-T) was added to the wells of assay plates previously coated with recombinant chIL-12p40 (rchIL-12p40) ex-COS. Specifically-bound antibody was detected with a secondary biotinylated goat anti-mouse IgG (GE Healthcare Life Sciences), followed by streptavidin-horseradish peroxidase (AbD Serotec, Kidlington, UK). Peroxidase activity was revealed with o-phenylenediamine (OPD) substrate (Sigma, Poole, UK), and absorbance was read at 492 nm in a SpectraMax 250 microplate spectrophotometer system (Molecular Devices, Sunnyvale, CA, USA).

A capture ELISA was developed using the anti-chIL-12p40 mAbs described in this paper. Falcon Microtest III flexible assay plates (Becton-Dickinson, Oxnard, CA, USA) were coated by overnight incubation at 4 °C with capture mAbs (1–5  $\mu$ g/ml in PBS, 50  $\mu$ l/well). Plates were washed three times with PBS-T, and then blocked with 100  $\mu$ l/well PBS-T containing 1% BSA (Sigma) for 2 h. After a further three washes in PBS-T, 50  $\mu$ l/well recombinant or native IL-12-enriched culture supernatant were added, and incubated for 2 h at RT. Unbound protein was removed by washing with PBS-T, biotinylated detecting mAb was added (0.5–1  $\mu$ g/ml in PBS, 50  $\mu$ l/well) and plates were incubated for 1 h at RT. They were then washed three times, prior to the addition of 50  $\mu$ l/well of streptavidin-horseradish peroxidase (AbD Serotec) diluted 1:500 in PBS-T and incubation for 1 h at RT. After washing three times in PBS-T, peroxidase activity was developed by adding 75  $\mu$ l/well of Ready-to-use tetramethylbenzidine (TMB) substrate (Sigma) for 10–15 min at RT. The reaction was stopped by the addition of 75  $\mu$ l/well of 0.5 M H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450 nm in a SpectraMax 250 microplate spectrophotometer system (Molecular Devices).

Dot blots were performed by dotting recombinant cytokines or control samples onto Hybond C<sup>+</sup> nitrocellulose membrane (GE Healthcare Life Sciences) and allowed to air-dry. Blots were then incubated in blocking solution (5% skimmed milk powder in PBS) overnight, at RT. After washing three times in PBS-T, they were incubated with the anti-chIL-12p40 mAbs, diluted 1:10 in blocking buffer, for 1 h at RT. Primary antibody was removed by washing three times in PBS-T and blots were then incubated with rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (DAKO, Glostrup, Denmark) diluted 1:1000 in PBS, for 1 h at RT. After a further five washes in PBS-T, detection was carried out using enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences), according to the manufacturer's instructions.

### 2.4. Isolation of bone marrow-derived macrophages and stimulation to produce IL-12

Femurs of 5- to 6-week-old Rhode Island Red chickens were removed *post-mortem* and isolated from the surrounding muscle tissue using sterile instruments. Both ends of the bone were cut off and the marrow flushed out with RPMI 1640 medium, using a 21G needle and syringe. Clumps of marrow were disaggregated by pipetting and large debris excluded by passing the suspension through a 100  $\mu$ m cell strainer. The cell suspension was



washed in RPMI 1640, resuspended at  $5 \times 10^6$  cells/ml in complete media (RPMI 1640 containing 5% chicken serum (Invitrogen), 1% L-glutamine, 1 U/ml penicillin and 1  $\mu$ g/ml streptomycin) supplemented with rchGM-CSF (ex-COS, 1:100; Avery et al., 2004), plated out in 24-well plates and incubated for 7 days at 41 °C, 5% CO<sub>2</sub>. The adherent monolayer of cells were re-fed every 2–3 days, by carefully removing non-adherent cells and spent media, and replacing with fresh complete media containing rchGM-CSF. On day 7 of culture, cells were stimulated with a variety of compounds to induce IL-12 expression: ConA (Sigma) (1  $\mu$ g/ml); LPS (*E. coli* serotype 055:B5 (Sigma), 4  $\mu$ g/ml); CpG DNA (pCI-neo plasmid DNA (Promega), 10  $\mu$ g/ml); soluble CD40 ligand (CD40L or CD154) fusion protein (3  $\mu$ g/ml) (Tregaskes et al., 2005); polyI:C (Sigma) (30  $\mu$ g/ml). All were used in the presence or absence of rchIFN- $\gamma$  (ex-COS, 1:200; Lawson et al., 2001). Cells were stimulated for 4.5 h and 18 h, at which point supernatants were collected for assaying by capture ELISA and cells harvested for RNA isolation using an RNeasy mini kit (QIAGEN, Crawley, UK).

### 2.5. Real-time quantitative RT-PCR

IL-12p35 and IL-12p40 mRNA levels were quantified by TaqMan qRT-PCR, using a well-described method (Avery et al., 2004; Eldaghayes et al., 2006; Rothwell et al., 2004). Primers and probes for IL-12p35, p40 and 28S RNA-specific amplification have been described previously but for clarity their sequences are as follows: IL-12p35 forward primer, TGGCCGCTGCAAACG; reverse, ACCTCTCAAGGGT-GCACTCA; probe, CCAGCGTCCTCTGCTTCTGCACCTT. IL-12p40 forward primer, TGGGCAAATGATACGGT-CAA; reverse, CAGAGTAGTTCTTGCCTCACATTTT; probe, CTGAAAAGCTATAAAGAGCCAAGCAAGACGTTCT. 28S forward primer, GGCGAAGCCAGAGGAACT; reverse, GACGACCGATTGACGTC; probe, AGGACCGCTACGGAC-CTCCACCA. Probes were all labelled with the fluorescent reporter dye FAM at the 5' end and with the quencher TAMRA at the 3' end. The RT-PCR was performed using the TaqMan Fast Universal PCR master mix and one-step qRT-PCR master mix reagents (Applied Biosystems, Warrington, UK). Amplification and detection of specific products were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems) with the following cycle profile: one cycle of 48 °C for 30 min and 95 °C for 20 s, and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Results are expressed as 40-Ct, after normalising each sample using the Ct value for the 28S rRNA product for the same sample, as described previously (Avery et al., 2004; Eldaghayes et al., 2006; Rothwell et al., 2004).

### 2.6. Immunocytochemistry

Rhode Island Red chickens were infected at three weeks of age with  $10^{1.7}$  EID<sub>50</sub> of the virulent infectious bursal disease virus (IBDV) strain F52/70 (Bygrave and Faragher, 1970) by the intranasal route in a total volume of 100  $\mu$ l (Eldaghayes et al., 2006). Bursal tissue from 5 infected and 3 age-matched uninfected control birds at 4 days

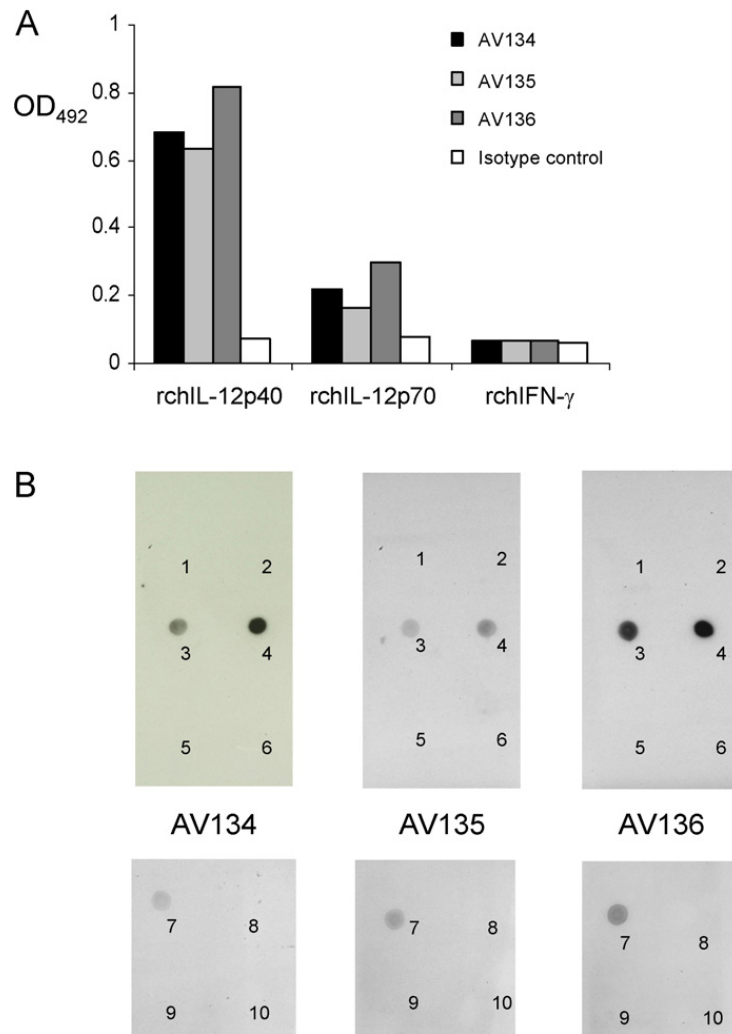
post-infection was snap-frozen in OCT (Sakura Finetek, Thatcham, UK). Frozen sections (6–8  $\mu$ m) of the bursa of Fabricius were cut using a cryostat, placed onto glass slides, fixed in acetone for 10 min and air-dried. Staining was carried out using a Vectastain® ABC anti-mouse IgG HRP staining kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions, using the anti-chIL-12p40 mAbs (diluted 1:10 in PBSa, 1% bovine serum albumin, 0.1% sodium azide) (or appropriate isotype control mAbs) as the primary antibody. Staining was visualised by incubation with peroxidase substrate solution (Vector® NovaRED™) and subsequent counter-staining with haematoxylin, to produce red staining for the target cells and blue staining for the background.

## 3. Results and discussion

This paper describes the production and characterisation of murine anti-chIL-12p40 mAbs, following successful DNA immunisation of mice. Of the five mAbs generated, two have been used to successfully develop an IL-12-specific capture ELISA, as described below.

Following three DNA immunisations, two out of six mice showed good antibody responses, as measured by indirect ELISA, and one was used for a fusion. Fusion plates were also screened by indirect ELISA, and five IL-12p40-specific antibody-secreting hybridomas were selected for cloning, expansion and characterisation. Two mAbs (AV114 and AV140) were IgM and were not characterised further. The three remaining mAbs (AV134, AV135 and AV136) were IgG1 isotype, and all recognise rchIL-12p40 and rchIL-12p70 in both indirect ELISA (Fig. 1A) and dot blots (Fig. 1B). Fig. 1B shows that the mAbs are specific for chIL-12 and do not cross-react with recombinant mammalian (human or bovine) IL-12, nor with supernatant from COS cells transfected with pCI-neo alone, or pCI-neo expressing chIFN- $\gamma$ . The three mAbs only bind to IL-12p40 in its native form, but do not recognise denatured antigen (Fig. 1B).

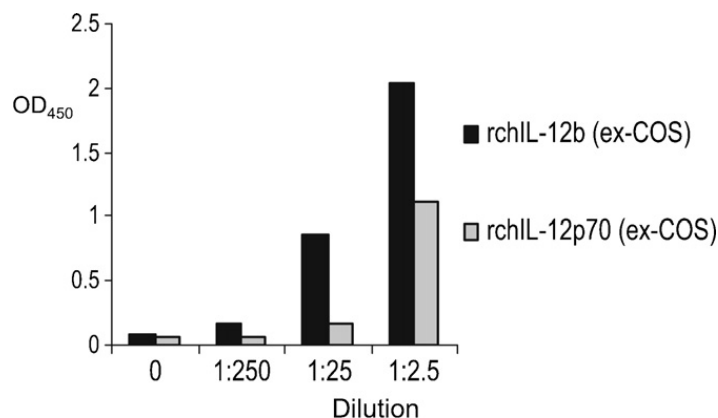
Various combinations of the three IgG1 mAbs, at different dilutions, were used to develop an IL-12 capture ELISA. Since the three mAbs were of the same isotype, unconjugated mAbs were used as capture Abs, whilst biotinylated mAbs were used as the detecting mAbs. All three mAbs worked in all possible combinations, with the optimal pair being AV136 (capture mAb, at 2  $\mu$ g/ml) and biotinylated-AV134 (detecting mAb, at 0.5  $\mu$ g/ml), resulting in the greatest sensitivity. The capture ELISA readily detected recombinant chIL-12p40 and chIL-12p70 derived from COS cells (Fig. 2), in a dose-dependent manner, and was then used to identify "native" IL-12 expressed by stimulated primary macrophages *in vitro* (Fig. 3A). Bone marrow cells were cultured *in vitro* for 7 d in the presence of rchGM-CSF to generate primary macrophages, which were then stimulated with a variety of compounds known to induce IL-12 production in mammals, including TLR agonists such as LPS, CpG DNA, and polyI:C and host-related stimuli such as CD40L, with or without IFN- $\gamma$  priming. Cells and supernatants were harvested at 4.5 and 18 h post-stimulation (hps). RNA was isolated from the cells for subsequent analysis by qRT-PCR (Fig. 3B) and supernatants were assayed for IL-12 using the capture ELISA (Fig. 3A).



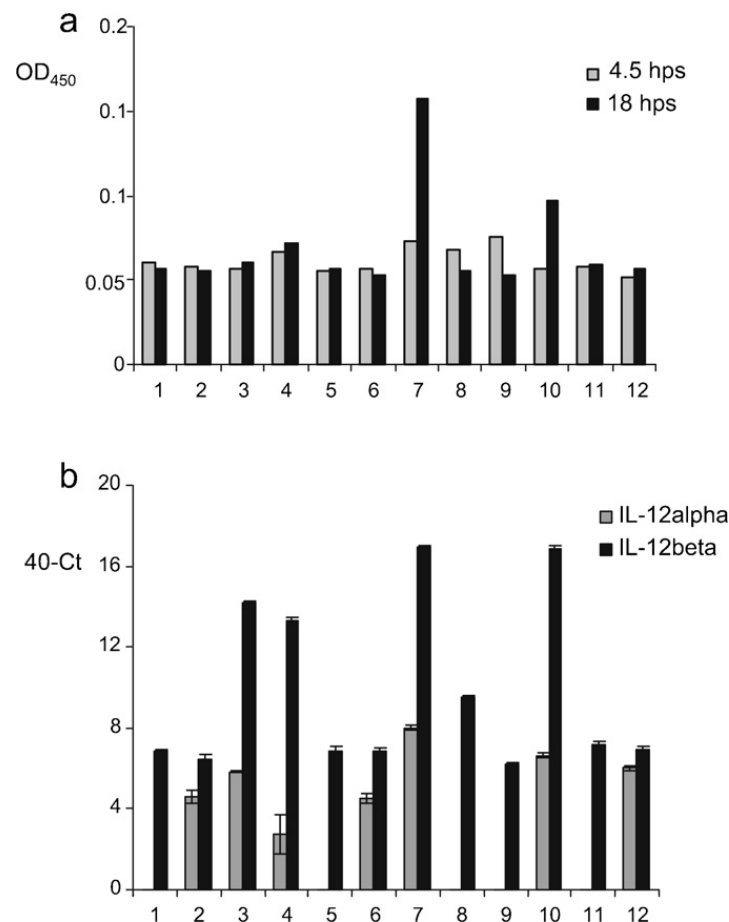
**Fig. 1.** Specificity of mouse anti-chicken IL-12p40 mAbs AV134, AV135 and AV136 demonstrated by (A) indirect ELISA against rchIL-12p40 and rchIL-12p70; rchIFN-γ was used as a negative control. (B) Dot blots showing specificity of the three mAbs against various sources of recombinant IL-12 – 1: pCI-neo (ex-COS); 2: rchIFN-γ (ex-COS); 3: rchIL-12p40 (ex-COS); 4: rchIL-12p70 (ex-COS); 5: rhIL-12 (100 μg/ml); 6: rbovIL-12 (ex-COS), and that the three mAbs recognise antigen in the native form; 7: rchIL-12p40 (ex-COS) in the native form; 8: rchIL-12p40 (ex-COS) heat-denatured; 9: pCI-neo (ex-COS) in the native form; 10: pCI-neo (ex-COS) heat-denatured.

Unsurprisingly, no IL-12 was detected in the culture supernatants harvested at 4.5 hps. However, IL-12 was detected in the supernatants harvested 18 hps from macrophages stimulated with LPS and IFN-γ, and to a lesser

extent in those stimulated with LPS and CD40L (Fig. 3A). Analysis of IL-12 mRNA expression by qRT-PCR (Fig. 3B) showed significant up-regulation of IL-12p35 and IL-12p40 in the same samples at 4.5 hps. IL-12p40 mRNA was also



**Fig. 2.** Capture ELISA using mouse anti-chicken IL-12p40 mAb AV136 as the capture Ab (at 2 μg/ml) and biotinylated-anti-chIL-12p40 mAb AV134 (at 0.5 μg/ml) as the detecting Ab. Capture ELISA detects both rchIL-12p40 (ex-COS) (black bars) and rchIL-12p70 (ex-COS) (grey bars) at different dilutions.

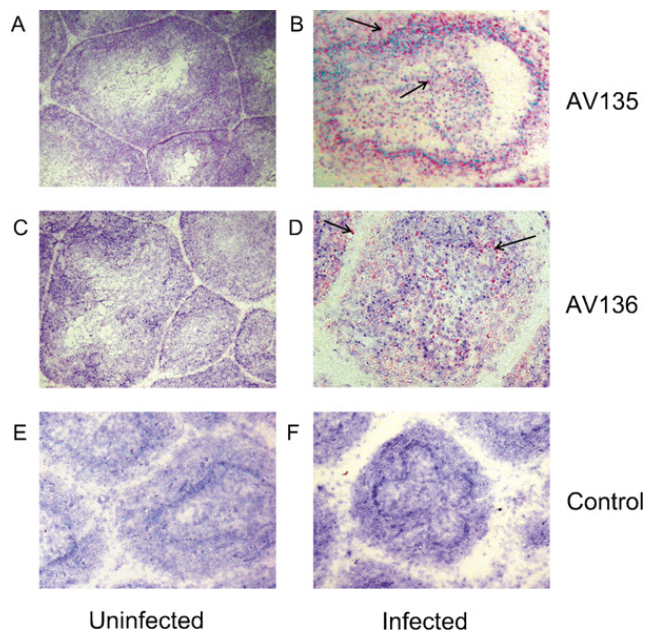


**Fig. 3.** Expression of IL-12 following stimulation of primary bone marrow-derived macrophages after 7 days of culture in the presence of rchGM-CSF. (A) IL-12 protein expression detected in the culture supernatant by capture ELISA (using mAbs AV136 and AV134) at 4.5 and 18 h post-stimulation (hps) (grey and black bars, respectively). (B) IL-12p35 (grey bars) and IL-12p40 (black bars) mRNA expression detected by qRT-PCR at 4.5 hps. 1: media; 2: ConA (1 µg/ml); 3: LPS (*E. coli* serotype 055:B5, 4 µg/ml); 4: CpG DNA (pCI-neo, 10 µg/ml); 5: soluble chCD154 fusion protein (3 µg/ml); 6: polyI:C (30 µg/ml); 7: LPS (4 µg/ml) + recombinant chIFN-γ (ex-COS, 1:200); 8: CD154 (3 µg/ml) + rchIFN-γ (1:200); 9: polyI:C (30 µg/ml) + rchIFN-γ (1:200); 10: CD154 (3 µg/ml) + LPS (4 µg/ml); 11: CD154 (3 µg/ml) + polyI:C (30 µg/ml); 12: polyI:C (30 µg/ml) + LPS (4 µg/ml). qRT-PCR results are expressed as mean corrected 40-Ct values  $\pm$  SE. The data shown are representative of three independent experiments.

up-regulated in macrophages stimulated with LPS alone or CpG DNA when compared to unstimulated macrophages, and although IL-12p35 was also detected in those samples, it was not as highly expressed as in the LPS + IFN- $\gamma$ - and LPS + CD40L-stimulated samples. IL-12p35 mRNA was not detected in cells cultured in media alone, in the presence of CD154 or polyI:C alone, or in the presence of CD154 and rchIFN- $\gamma$  or polyI:C. By 18 hps, IL-12p40 mRNA expression was no longer significantly up-regulated, whilst IL-12p35 was still up-regulated in some cells (data not shown). Biologically active IL-12 (IL-12p70) can only be produced in cells that co-express both p35 and p40 subunits (Gubler et al., 1991), and in mammals IL-12p35 is ubiquitously and constitutively expressed, so it is the more tightly regulated and restricted expression of IL-12p40 that determines a cell's ability to produce functional IL-12 (Gately et al., 1998). In contrast, in the chicken, IL-12p40 is ubiquitously expressed in a wide variety of tissues (Balu and Kaiser, 2003), whilst expression of IL-12p35 is more tightly controlled (Balu, 2005; Degen et al., 2004), thus determining the production of the functional p70 molecule. The capture ELISA data (Fig. 3A), in concert with the qRT-PCR data (Fig. 3B), indicates that it is both the expression of p35

and the abundance of p40 that determines the amount of IL-12p70 produced. We have shown that, as in mammals, effective induction of chIL-12 *in vitro* requires at least two different signals (Snijders et al., 1998), specifically bacterial LPS (activating the TLR4 pathway) along with an 'endogenous' signal such as engagement of the CD40-CD40L signalling pathway or co-stimulation via IFN- $\gamma$ .

Using qRT-PCR, we had previously shown that IL-12p35 mRNA expression is up-regulated in the bursa two to four days post-infection (dpi) with virulent IBDV F52/70, whilst IL-12p40 is constitutively expressed in both uninfected and infected birds (Eldaghayes et al., 2006). Bursal sections (4 dpi) from the same IBDV-infection experiment (Eldaghayes et al., 2006) were stained with the anti-chIL-12p40 mAbs described herein, to investigate their utility in immunocytochemistry. Clear positive staining of IL-12-producing cells (using both mAbs, AV135 and AV136) was seen in the bursae of infected birds (Fig. 4B and D) when compared to bursal sections from age-matched control birds (Fig. 4A and C). Positively stained cells are mainly seen in the cortex of the bursa and at the cortico-medullary junction, with some IL-12-positive cells in the medulla, as well as in the interfollicular tissue. IBDV infection results in gross bur-



**Fig. 4.** Intracellular staining of cryosections of the bursa of Fabricius with anti-chIL12 mAbs by immunocytochemistry. (A, C and E) Sections of bursa from uninfected birds stained with anti-chIL-12p40 mAbs AV135, AV136 or an isotype control mAb, respectively. (B, D and F) Bursal sections from birds infected with the virulent infectious bursal disease virus strain F52/70 at 4 days post-infection, stained with AV135, AV136 or an isotype control mAb, respectively. Black arrows indicate selected positively stained (red) cells. Sections were counterstained with haematoxylin.

sal damage, caused by significant B lymphocyte depletion within the follicles; there is an influx of T cells, and migration of some mononuclear phagocytes into the damaged bursal follicles (Vervelde and Davison, 1997), which may be responsible for the production of IL-12 within the damaged bursa. Staining with the isotype control mAbs did not show any positive staining (Fig. 4E and F) in sections from either the uninfected or the infected birds.

We presume these mAbs are recognising IL-12p70, as IL-12p40 is constitutively expressed in the chicken, yet no staining was seen in the bursae of uninfected control birds. The observed lack of binding in uninfected bursal tissues may reflect a defect in full protein translation and production in uninfected animals, perhaps indicating that the bioactive protein is only produced in response to IBDV infection. However, in mammals IL-23 is a heterodimer of IL-23p19 and IL-12p40. Although chIL-23p19 has yet to be described, we cannot rule out that these mAbs could be recognising chIL-23.

In summary, the data presented here show that the mAbs raised against chicken IL-12p40 can be used to detect natural chicken IL-12 in a capture ELISA, allowing us for the first time to specifically measure this pivotal Th1-driving cytokine at the protein level in the chicken. The mAbs have been successfully used to detect IL-12-positive cells in frozen sections using immunocytochemistry, and may, with further optimisation, be suitable for use in flow cytometry and ELISpot assays. Further characterisation is required to ascertain whether the mAbs are capable of neutralising the bioactivity of chIL-12 or if they work in Western blot.

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